A novel marker of early epidermal differentiation: cDNA subtractive cloning starting on a single explant of *Xenopus laevis* gastrula epidermis

OLEG L. VASILIEV[1]*, SERGEY A. LUKYANOV[1], ALEXANDER V. BELYAVSKY[2], OLGA V. KAZANSKAYA[1], and ANDREY G. ZARAISKY[1]

[1]*Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, and* [2]*Institute of Molecular Biology, Russian Academy of Sciences, Moscow , Russia*

ABSTRACT To understand the molecular mechanism underlying in the earliest steps of the embryonic ectoderm subdivision into epidermis and neuroectoderm, it would be important to isolate differentially expressed genes in presumptive neuroectoderm and epidermis at the gastrula stage, the period of the divergence of the two adjacent ectodermal compartments. Meanwhile, the most direct approach for such a task, i.e. subtractive enrichment of cDNA from neuroectodermal and epidermal explants with differentially expressed gene sequences, was difficult to realize because of the high number of explants needed for this technique. In the present paper we report a novel effective and quite simple method of cDNA subtractive enrichment, based on amplification of cDNA *in vitro* by polymerase chain reaction (PCR) and allowing to use a very small amount of initial cDNA samples. With this method we have cloned cDNA of a novel gene of *Xenopus laevis*, which was named XEP-1 for its specific expression in the presumptive epidermis starting from the midgastrula stage.

KEY WORDS: *XEP-1 gene, presumptive epidermis, neuroectoderm, amphibians*

Introduction

Embryonic ectoderm in the Amphibia is subdivided into neuroectoderm and epidermis during gastrulation under the influence of dorsal mesoderm on the overlying part of ectoderm. As a result, the dorsal part of the ectoderm becomes neuroectoderm, while another, ventral part develops as epidermis (Spemann, 1938; Saxen and Toivonen, 1962; Slack, 1983). To understand the molecular mechanism of this subdivision, it is important to identify genes which are differentially expressed in the developing epidermis and neuroectoderm as early as possible. Several such genes, specifically expressed in the neuroectoderm, but not in epidermis, have been recently identified (Kintner and Melton, 1987; Sharpe *et al.*, 1987,1989; Sharpe, 1988; Brivanlou and Harland, 1989; Ruiz i Altaba and Jessell, 1992). Meanwhile less genes are known which could be used as markers for early epidermal differentiation (Jamrich *et al.*, 1987; Savage and Phillips, 1989). Bearing this in mind, we have undertaken an attempt to clone novel genes specifically expressed just in the presumptive epidermis cells of *Xenopus laevis* early embryo.

Isolation of *a priori* unknown genes differentially expressed in two extremely small samples of tissue such as neuroectodermal and epidermal explants (each not exceeding 2-3 thousands of cells), is a common difficulty for investigations on cell differentiation during embryonic development. Subtractive enrichment based on initial cDNA amplification by polymerase chain reaction (PCR) seems to be the most direct approach to such a task. In a previous paper we described a version of PCR-based subtractive cloning which makes it possible to use even single explants of embryonic tissues (Zaraisky *et al.*, 1992). As a result of differential screening of subtracted cDNA library from the *Xenopus laevis* presumptive neuroectoderm by probes enriched with neuroectoderm or epidermis specific sequences, we isolated a previously unknown homeobox gene, *XANF-1*, which was specifically expressed within the anterior presumptive neuroectoderm (Zaraisky *et al.*, 1995).

In the present work we have performed differential screening of subtracted library enriched with epidermis-specific cDNAs using the same initial cDNA samples, from *Xenopus* neuroectoderm and epidermis. The work has two main novelties. First, we have developed a simpler, PCR-based method of cDNA enrichment which may be used in many fields of developmental biology. Second, we have cloned cDNA of a novel gene which is specifically expressed in the *Xenopus* epidermis cells at the beginning of their determination, i.e., the midgastrula stage.

***Address for reprints:** Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, ul. Miklukho-Maklaja 16/10, V-437 Moscow 117871, Russia. FAX: 7 (095) 336-11-55. e-mail:ocher@ibch.siobc.ras.ru

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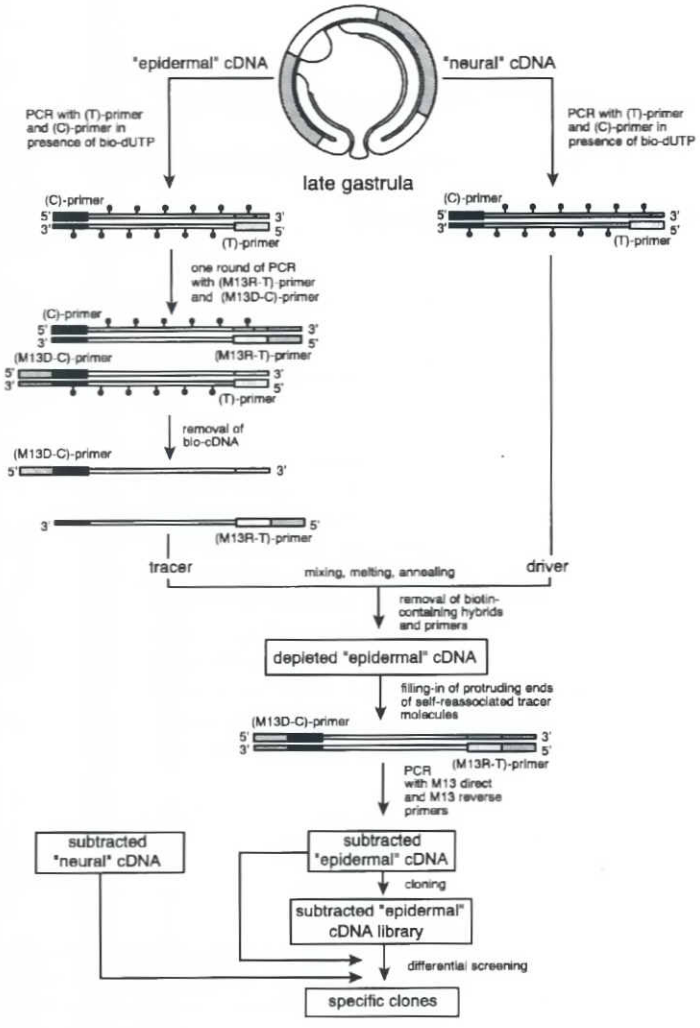

Fig. 1. A scheme of subtractive cloning of epidermis-specific sequences, *(see text for details).*

Results

Subtractive cloning of epidermis-specific cDNAs

We have utilized the already obtained cDNA samples (Zaraisky *et al.*, 1992). These samples were synthesized on the basis of the total RNA isolated from two explants of presumptive neuroectoderm («neural» sample) and epidermis ("epidermal" sample), taken from the midgastrula embryos of *Xenopus laevis*. Each of the explants contained no more than 2000-3000 cells. The procedure of cDNA amplification, including removal of (T)-primer and PCR with (C)- and (T)-primers, has already been described (Belyavsky *et al.*, 1989).

The subtraction procedure is illustrated in figure 1 on the example of «epidermal» cDNA subtraction. Initially the «epidermal» and «neural» samples were obtained in a biotinylated form by performing PCR with (T)- and (C)- primers in the presence of bio-11-dUTP. Unbiotinylated «epidermal» DNA strands, elongated only at the 5' end with M13 direct and reverse primers sequences, were then obtained by an additional round of PCR with two long primers and purification from biotinylated cDNA strand with heat-denaturation, followed by two standard streptavidin-phenol-chloroform extraction (Barr and Emanuel, 1990). At the next step 100 ng of the purified unbiotinylated "epidermal" cDNA (tracer) were mixed with a 20 fold excess of biotinylated "neural" cDNA (driver), and after melting and reannealing biotin-coutaining hybrids, i.e., common sequences were removed. Once depleted, the tracer was passed through another round of subtraction with the same amount of the driver. Finally, this twice depleted tracer cDNA was incubated for 2 min at 72° with Taq-polymerase (to fill in 5' protruding ends) and amplification by PCR was performed in the same mixture with M13 direct and reverse primers. At this step only self-reassociated tracer ("epidermal") cDNA molecules had an opportunity to be supplied with M13 primers on both ends. Since, only these, rather than any other double- or single-stranded cDNA molecules, could be amplified in geometrical progression during PCR.

After reamplification with (T)- and (C)- primers, a part of subtracted tracer was cloned into pGEM1 vector by the procedure described elsewhere (Belyavsky *et al.*, 1989). After radioactive labeling, another part of the tracer was used as one of the two probes for subsequent differential screening. The other probe was obtained on the basis of "neural" cDNA subtracted by the "epidermal" one, which was used in this case as a driver.

Validity of the subtraction procedure

To verify the validity of this subtraction protocol we have tested enrichment of the natural internal standard presenting in the "neural"

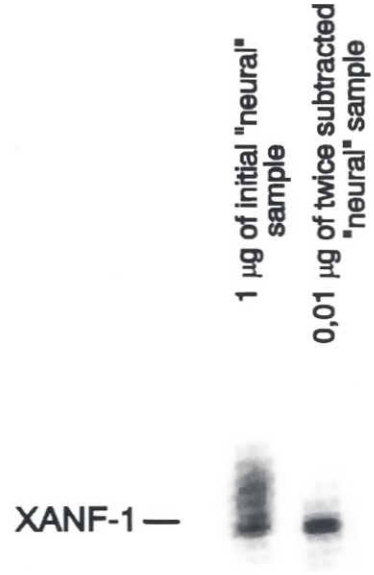

Fig. 2. Southern analysis of enrichment of the twice-subtracted «neural» sample with a neural specific cDNA, XANF-1. *«Neural» sample was blotted on a nitrocellulose filter before and after two rounds of subtraction and hybridized with ^{32}p labeled probe for XANF-1. Note that only 10 ng of the twice subtracted sample displayed the same intensity of hybridization signal as did 1 μm of unsubtracted cDNA.*

sample, cDNA of homeobox gene XANF-1. As we have already found (Zaraisky *et al.*, 1992), this gene is expressed at the middle-late gastrula stage specifically in the presumptive neuroectoderm. In the experiment, we have hybridized a labeled probe for XANF-I with the "neural" cDNA which was blotted on a nitrocelulose filter before and after two rounds of subtraction (Fig. 2). Densitometric analysis showed that there was more than 100 times strong signal in the case of enriched sample.

cDNA of a novel epidermis-specific gene, XEP-1

Hundred clones from subtracted "epidermal" library were analyzed on parallel replicas using "epidermal" and "neural" probes. As a result, we have identified one clone containing cDNA (657 n.p.) of a novel epidermis-specific gene (GenBank accession L10987), which we called *XEP-1* (for *Xenopus* epidermis). As was shown by Northern blotting of gastrula poly(A)$^+$ RNA, this cDNA corresponded to the full-length (or almost full-length) sequence of XEP-1 mRNA (not shown).

The longest open reading frame of the insert, starting from methionine, encodes 136 amino acids (Fig. 3). Specific features of the XEP-1 protein structure are a relatively low molecular weight (14 983 dalton) and high amount of cysteine residues (seven).

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  1  attccactgagcaaggttgtcactgggagagttttcatctggggaatgttattgctttca

 61  aagtagcctgacttgggactcaacggaatcagggactactgtgtcagctgctgtggttcc

121  acttcggctaaaactATGAGGGTACTTCCCTTCCTGGCTCTCACTGTCGCCTGCGTCTTC
  1                  M  R  V  L  P  F  L  A  I  T  V  A  C  V  F

181  AGCACTGGGTCTGGAGATAATGGAGATTGCTTCTTCTATGATCAGGTCTACAAAGATGGA
 17   S  T  G  S  G  D  N  G  D  C  F  F  Y  D  Q  V  Y  K  D  G

241  GATACTATTAAATATGATTGCCAAATCTGTGAATGTACCAATGGAAGAATTACAGACTGT
 37   D  T  I  K  Y  D  C  Q  I  C  E  C  T  N  G  R  I  T  D  C

301  GCACGTGATGCGAACTGCATCTTTAAAAAGGTGGCTACACCTGAGCAGAATACTGATGCG
 57   A  R  D  A  N  C  I  F  K  K  V  A  T  P  E  Q  N  T  D  A

361  ATAGTCTATGATGATGATGATCCAAATATCATTGAAAGCAGCGAGAGCAAGGGGAATGTT
 77   I  V  Y  D  D  D  D  P  N  I  I  E  S  S  E  S  K  G  N  V

421  CGAGCGAAACGGGCTGCGTCTGGGGACATAAGAAGAACCCCAGTGCTTATGAGACAAACA
 97   R  A  K  R  A  A  S  G  D  I  R  R  T  P  V  L  M  R  Q  T

481  ATAAATTCAAGCCAAACCAAAGCAACCGTAATGATAATGATGACTCAGCAAGGAACTCAA
117   I  N  S  S  Q  T  K  A  T  V  M  I  M  M  T  Q  Q  G  T  Q

541  GGAtagtaacaaagggaaaggacagaaaatcaacaatgtattgtcaaaggattcatctga
      G  *  *

601  agaaaatgcatctaaagaaaaatggagtcatgaaaagaatgataaaatgaagacaaaag
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Fig. 3. Nucleotide and deduced amino acid sequences of XEP-1 cDNA. *The ATG at the position 126-128 represents the putative start codon of the XEP-1-encoded protein. Leader peptide is underlined. Asterisk indicates the termination codon.*

Another interesting feature is N-terminal amino-acid sequence, MetArgValLeuProPheLeuAla IleThrValAlaCysValPheSerThrGlySerGly (Fig. 3), enriched with hydrophobic amino acids, well corresponding to the leader peptide structure of the secreted proteins (Gunnar von Heijne, 1988).

Spatial and temporal distribution of XEP-1 transcripts during early Xenopus development

Spatial-temporal pattern of *XEP-1* expression was analyzed by reverse-PCR and *in situ* hybridization. The onset of expression coincided with the beginning of gastrulation and progressively increased throughout gastrulation and neurulation. *XEP-1* mRNA was not found in the pregastrula embryos and in the ovaries (Fig. 4).

We could not detect XEP-1 expression in any other embryonic tissue, except ectoderm. More careful analysis has shown that before the early midgastrula stage (stage 11) *XEP-1* mRNA was presented in all regions of the ectoderm. However, already at the end of gastrulation and later expression of this gene was detected only in the epidermis (Fig. 5).

Discussion

Despite numerous strategies of subtractive cDNA cloning have been developed (Sargent, 1987; Duguid and Dinauer, 1990; Timblin *et al.*, 1990; Hara *et al.*, 1991; Swaroop *et al.*, 1991), revealing low-scale differences between two natural sets of cDNA sequences remains a formidable task because of the high complexity of the cDNA populations, which increases a dangerous to lost differentially distributed sequences due to the driver "pollution". The general idea of our method is based on the utilization of long T and C-primers, consisting of two parts, which permits one to make selective amplification of the differentially distributed tracer sequences even in the presence of a large amount of driver.

Additionally we have used the approach proposed by Lisitsyn *et al.*, (1993a,b) for genomic subtraction. They combined subtractive enrichment with the kinetic one. The latter is based on the second-order kinetic of reassociation of complementary strands, which gives unlinear advantage in the rate of reassociation to the molecules appearing in excess as a result of subtraction. Presumably these molecules represent specific sequences of the tracer. Theoretically if after subtraction tracer-specific cDNAs have been enriched N times relative to other molecules, after subsequent kinetic enrichment (which consists of melting and short-term reannealing of the tracer), in a newly forming double-stranded hybrid population these molecules would be present N^2 times relative to other sequences present as duplex DNA (Wieland *et al.*, 1990). The problem lies only in purification of the population of double-stranded DNA.

Unlike Lisitsyn and his colleagues, who used ligation of the specific adapters to purify kinetically enriched tracer hybrids

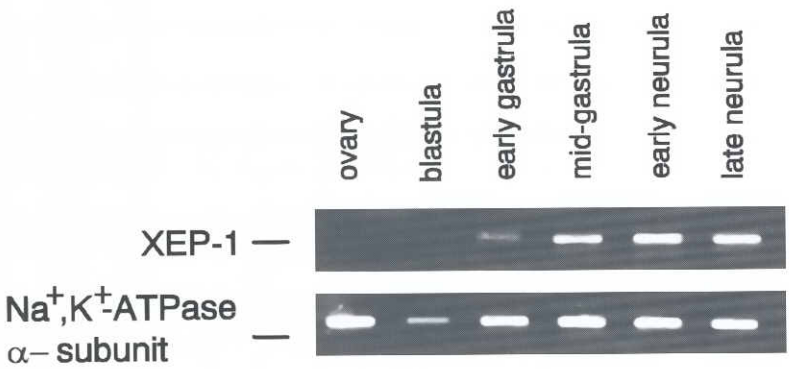

Fig. 4. Temporal pattern of *XEP-1* expression. *Total RNA samples were reversed transcribed and subjected to 31 cycles of PCR with oligonucleotide primers for XEP-1 and for Na^+, K^+ -ATPase α-subunit (as a control). PCR products were separated by eletrophoresis in 1.5% agarose gel with ethidium bromide and were photographed. Developmental stages: blastula (stages 8-9), early gastrula (stages 10-10.5), mid gastrula (stages 11-12), early neurula (stages 13-14), late neurula (stages 18-20).*

throwing away all drivers and unreannealed tracers, we used a simpler procedure with (T)- and (C)-primers elongated by sequences of M13 direct and reverse primers. After subtractive hybridization only self-reannealing tracer molecules, which contain these elongated primers at 5´ ends, could be filled in at 3' ends and amplified by PCR with M13 direct and reverse primers. This approach permits one to simplify the protocol, avoiding additional step of primers ligation.

The validity of our subtraction protocol was confirmed, firstly, by the analysis of enrichment in the "neural" probe of previously cloned neuroectoderm-specific homeobox gene, *XANF-1*, and, secondly, by the cloning of a novel epidermis-specific gene, *XEP-1*. As was shown by reverse-PCR and *in situ* hybridization, at the midgastrula stage transcripts of the new gene are present only in the presumptive epidermis cells, but not in cells of the bordering neuroectoderm. Meanwhile, we could not find any differences between the presumptive neuroectoderm and epidermis in the *XEP-1* RNA distribution at the beginning of gastrulation. These data suggest that changes in the pattern of XEP-1 expression could follow the well-known strategy of many genes operative in embryogenesis: narrowing of initially wide expression zone. We propose that in the case of *XEP-1* such narrowing may be due to inhibition of its expression in the presumptive neuroectoderm by the neural inducer-dorsal mesoderm.

The same type of spatial-temporal expression pattern (postgastrulation disappearance of transcripts in the presumptive neuroectoderm) was shown earlier for two other epidermis-specific genes of *Xenopus laevis: cytokeratin XK81* and *Epi1* (Jamrich *et al.*, 1987; Savage and Phillips, 1989).

No related sequences of XEP-1 were found in the GenBank Data Library. Thus, now it is difficult to make a conclusion about possible functions of the protein coded by this gene. Meanwhile, taken into account its low molecular weight and presence on the N-terminal amino-acid sequence, which well corresponds to the leader peptide structure of the secreted proteins, one may hypothesize that *XEP-1* could be a growth factor. Such possibility would be very intriguing since there were no indications of growth factors, which would be expressed during Amphibian gastrulation specifically in the epidermis cells.

Materials and Methods

Subtraction methodology and differential screening

Samples of cDNA amplified by PCR with (T)- and (C)-primers have already been obtained (Zaraisky *et al.*, 1992) from single explants of the presumptive epidermis ("epidermal" sample) and neuroectoderm ("neural" sample) taken at the late gastrula stage [stage 12 (Nieuwkoop and Faber, 1975)].

To obtain biotinylated "neural" and "epidermal" cDNA, 5 ng DNA from each sample were passed through additional 20 rounds of PCR in a reaction mixture (1 ml) containing 10 mM Tris-HCl (pH 8,3), 50 mM KCl, 4mM $MgCl_2$, 0.01% (w/v) gelatine, 0.2 mM of each dNTP, 0.05 mM Bio-11-dUTP, 500 pmole of (T)-primer (5'-$GGGAGGCCC(T)_{13}$), 500 pmole of (C)-primer (5'- $AAGGAATT(C)_{13}$) and 20 units of Taq-polymerise (Perkin-Elmer-Cetus). The reaction cycle consisted of denaturation at 96°C for 30 sec, annealing at 60°C for 30 sec, and elongation at 72° for 3 min. After phenol-chloroform extraction and two times precipitation with ethanol, 300 ng of each "neural" and "epidermal" biotinylated cDNA each were purified

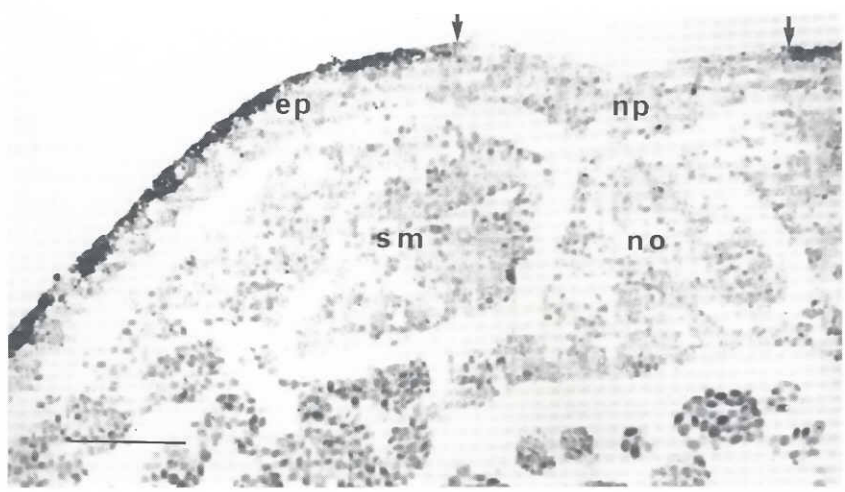

Fig. 5. In situ hybridization with XEP-1 digoxygenin-labeled probe. *Whole-mount in situ hybridization was performed with albino-embryos collected at the early neurula stage (stage 14). Treated embryos were embedded in paraffin, sectioned and stained with Eosin. Note that hybridization signal presents only in the outer epidermal cells, but not in the bordering neural plate cells. ep, epidermis; np, neural plate; no, notochord; sm, somite. Arrows indicate the borders between epidermis and neural plate. Bar: 50 μm.*

from (T)- and (C)-primers by electrophoresis in low-melt agarose as described elsewhere (Belyavsky *et al.*, 1989).

To obtain "neural" and "epidermal" cDNAs, elongated on the 5' ends with additional sequences of phage M13 reverse and direct sequencing primers, 200 ng of each biotinylated cDNA were passed through a single round of PCR in 100 μl of a reaction mixture containing (M13Reverce-T)-primer (5'-GAGGAAACAGCTATGACCCGGGAGGCCCC$(T)_{13}$) and (M13Direct-C)-primer (5'- GTAAAACGA CGGCCAGTGAATTCCTTAA$(C)_{13}$) instead of (T)- and (C)-primers. To remove biotinylated strands, cDNA samples were denaturated at 96° for 2 min, treated with streptavidin and subjected to phenol-chloroform extraction (Barr and Emanuel, 1990).

After purification from the primers by electrophoresis in low-melt agarose, 150 ng of "epidermal" cDNA elongated at 5' ends with M13-Direct and M13-Reverse sequences (tracer) were hybridized with 3 μg of biotinylated "neural" cDNA (driver) in 5 μl of a hybridization mixture containing 0.25 mM EDTA, 0.5 M NaCl, 0.05 M HEPES pH 7.2, 0,1% SDS, 10 μg east tRNA, 100 pmole (T)-primer and 100 pmole (C)-primer. Fifty μl of mineral oil were added to the hybridization mixture to avoid evaporation, and after denaturation at 95° for 2 min the mixture was incubated at 65° for 16 h. Thereafter 100 μl NTE buffer (10 mM Tris-HC1, pH 8.0, 0.5 M NaCl, 1 mM EDTA) were added and the mineral oil was extracted by chloroform. Biotin-containing hybrids were removed by adding 10 μg streptavidin with subsequent phenol-chloroform extraction (Barr and Emanuel, 1990). After precipitation with ethanol the subtracted tracer cDNA was again hybridized with 3 μg of driver for 10 h.

Depleted "epidermal" cDNA was amplified in 50 μl of PCR mixture with 20 pmoles of M13 direct primer (5'-GTAAAACGACGGCCAGT) and M13 reverse primer (5'-CAGGAAA CAGCTATGAC). After 30 rounds of PCR the reaction mixture was diluted up to 1000 times and subjected to additional 17 rounds of PCR with (T)- and (C)-primers.

Ligation of subtracted "epidermal" cDNA into pGEM1 vector was performed as described elsewhere (Belyavsky *et al.*, 1989).

For differential screening 100 individual clones from the subtracted library were replicated and hybridized with P^{32}-labeled probes synthesized by random priming of "epidermal" or "neural" subtracted samples.

DNA sequencing

XEP-1 cDNA was sub-cloned into an M13 vector and sequenced by the dideoxy-chain termination method (Sanger *et al.*, 1977) in both orientations. Three independent clones were sequenced. The sequence has accession number L10987 in the GenBank Data Library.

PCR analysis of gene expression

In all experiments PCR analysis was conducted for five-six individual explants obtained from different embryos. Total RNA samples were extracted using the standard guanidine isothiocyanate procedure (Chomczynski and Sacchi, 1987), reverse transcribed according to the standard protocol (Amersham) in a total volume of 20 ml and subjected to PCR with oligonucleotide primers for XEP-I (5' GACTTGGGACTCAACGGAATC and 5' GCCCGTTTCGCTCGAACAT) or Na^+,K^+-ATPase α-subunit (5' AACCGGGACCCTAACGCAGA and 5' ACGCTCAGGGGCTCCTTTCA (Verrey *et al.*, 1989)). The lengths of amplified fragments were: 348 base pair (bp) for *XEP-1* and 410bp for Na^+,K^+-ATPase α-subunit. PCR was performed in 100 μl of a buffer containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 4 mM MgCl, 0.01% (w/v) gelatine, 0.5 mM of each dNTP, 40 pmoles of each primer and 2 units Taq polymerase (Perkin Elmer Cetus). The reaction cycle consisted of denaturation at 96° for 30 sec, annealing at 58° for 30 sec, and elongation at 72°C for 30 sec. Thirty one cycle were performed in both cases. PCR products were separated by electrophoresis in 1.4% agarose gel containing 0.5 mg/ml ethidium bromide and photographed. For confirmation of band identity, each set of bands was subjected to Southern blotting analysis (10) with a P^{32} labeled probe to the corresponding gene sequence (data not shown). In order to check contamination by DNA, each RNA sample was subjected to PCR in the presence of all pairs of primers, but without reverse transcription.

Northern blot and in situ hybridization

Total RNA for Northern blot was extracted using the standard guanidine isothiocyanate procedure (Chomczynski and Scchi, 1987) from approximately 1000 embryos at the late gastrula-early neurula stage. Poly(A)$^+$ RNA was isolated according to the standard protocol (Maniatis *et al.*, 1982). Agarose electrophoresis and Northern blot hybridization were performed using 10 μg of the poly(A)$^+$ RNA in a slot as described elsewhere (Maniatis *et al.*, 1982).

Whole-mount *in situ* hybridization was performed using the albino embryo exactly as described in (Harland, 1991) with digoxygenin-labeled RNA antisense and sense (as control) probes. Treated embryos were embedded in paraffin, sectioned and stained with Eosin.

Manipulation with embryos

Xenopus laevis embryos were obtained as described elsewhere (Keller, 1975) and staged according to Nieuwkoop and Faber (1975). Jelly coats were removed manually in Niu-Twitty medium (Niu and Twitty, 1953). Embryos were operated, using an eye-surgery micro knife and a fused glass capillary on plastic dishes coated with 1% agarose. Tissue explants were cultured in Nui-Twitty medium.

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